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1: J Natl Cancer Inst. 1993 Feb 3;85(3):207-16. Related Articles, Links

Human renal carcinoma line transfected with interleukin-2 and/or interferon alpha gene(s): implications for live cancer vaccines.

Belldegrun A, Tso CL, Sakata T, Duckett T, Brunda MJ, Barsky SH, Chai J, Kaboo R, Lavey RS, McBride WH, et al.

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BACKGROUND: Combination therapy with systemically administered interleukin-2 (IL-2) and interferon alpha (IFN-alpha) has resulted in long-term objective remissions in 30% of patients with metastatic renal cell carcinoma (RCC), but toxic effects are clinically significant. PURPOSE: We have thus investigated an alternative therapeutic approach--continuous intratumoral production of IL-2 and/or IFN-alpha by a cytokine-transfected human RCC tumor cell line. METHODS: Plasmid vectors were used to transfect the R11 RCC line with the genes for human IL-2 and/or IFN-alpha by the calcium phosphate precipitation method. Biologic characteristics of the cytokine-transfected tumor cells were determined by assays of thymidine incorporation and cytotoxicity, fluorescence-activated cell-sorter analysis, Northern blotting, and in vivo studies in C3Hf/Sed/Kam mice rendered T-cell deficient. RESULTS: The transfected cell lines produced the following amounts of cytokine per 10(6) cells per day: R11-IL-2 (220 U), R11-IFN-alpha (10,240 U), and R11-IL-2 + IFN-alpha (95 U + 1270 U, respectively). Gamma irradiation did not eliminate cytokine secretion. Morphology and growth rates were identical to those for the parental R11 cell line, except for IFN-alpha-producing clones, which showed significant growth inhibition. All cytokine-producing cells demonstrated increased susceptibility to cell killing by peripheral blood leukocytes (PBL). IFN-alpha producers exhibited enhanced HLA antigen expression and suppressed c-myc messenger RNA expression; when cocultured in vitro, they induced similar changes in parental R11 cells. IL-2 producers could stimulate growth and cytotoxicity of naive (i.e., freshly isolated, uncultured) and activated PBL. All cytokine-producing cells lost their tumorigenicity, as evidenced by failure to grow in the T-cell-depleted mice. When co-injected at a local site but not at a distant site, these cells prevented growth of parental R11 cells.

Histologic examination of the injection sites revealed a substantial influx of macrophages. Intraperitoneal administration of IL-2 and/or IFN-alpha could not, however, prevent growth of the parental R11 tumors. CONCLUSION: Local production of high concentrations of IL-2 and IFN-alpha at the tumor site is more effective in preventing tumor growth than systemic administration. IMPLICATION: Continuous local delivery of cytokines via transfer of cytokine genes into tumor cells for use as live cancer vaccines is a novel strategy for manipulation of host-mediated antitumor immune response in patients with advanced RCC.

PMID: 8423625 [PubMed - indexed for MEDLINE]

1: Patol Pol. 1992;43(2):51-4.

Membrane-bound tumour necrosis factor alpha: immunocytochemical and ultrastructural studies of human monocytes and monocytic cell line and its induction by tumour cells in vitro.

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Monoclonal antibody against recombinant human tumour necrosis factor alpha (rTNF) was used for the immunochemical detection of TNF in human blood monocytes and monocytic cell line U 937. Cells stimulated with phorbol myristate acetate (PMA) showed strong surface but not cytoplasmic staining. Unstimulated cells demonstrated weak or no staining. At early time after stimulation (1-2h) a spot reaction was seen in the Golgi area of the cytoplasm of stimulated cells. Coculture of tumour cells with monocytes also resulted in the induction of membrane TNF. Ultrastructural studies confirmed TNF localization within the cell membrane. These results indicate that TNF can be detected within the cells by immunocytochemistry which may make feasible studies on TNF appearance in cellular infiltrates in the tissues.

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1: J Leukoc Biol. 1992 Mar;51(3):296-9. Related Articles, Links

Soluble TNF and membrane TNF expressed on CD4+ T lymphocytes differ in their ability to activate macrophage antileishmanial defense.

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In our studies of host defense against the intracellular parasite Leishmania major, we obtained evidence for a novel mechanism of macrophage activation for antimicrobial defense that involves direct cell contact between CD4+ T lymphocytes and Leishmania-infected macrophages. The mechanism is distinctive as it does not involve secretion of lymphokines but is apparently mediated by the membrane-anchored form of tumor necrosis factor (mTNF; approximately 50-60 kd) present on the surface of the effector T lymphocytes. Furthermore, it is not cytotoxic to the host cell and its expression is antigen specific and genetically restricted. We prepared a Leishmania-specific cloned T-T cell hybridoma line 1B6 (CD4+, TH1) that expresses membrane-bound TNF but does not secrete TNF or other macrophage activators. We now report that 1B6 cells can activate antileishmanial defense in inflammatory macrophages, whereas soluble recombinant murine TNF (sTNF) alone is unable to do so. On the other hand, both 1B6 cells and sTNF can act synergistically with recombinant murine interferon-gamma (IFN-gamma, a known soluble macrophage-activating factor) in activating antimicrobial defense and NO2- release. The effects of 1B6 alone and the synergistic effects of 1B6 and IFN-gamma or sTNF and IFN-gamma are arginine dependent. These results suggest that mTNF may be more efficient than sTNF in macrophage activation and that contact with effector CD4+ lymphocytes that express mTNF may be an important mechanism of host defense.

PMID: 1347312 [PubMed - indexed for MEDLINE]

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Vol. 86, pp. 3286–3290, May 1989
Immunology

Identification and characterization of a membrane-bound cytotoxin of murine cytolytic lymphocytes that is related to tumor necrosis factor/cachectin

(T-cell killing/perforin/cytotoxic T lymphocyte/cell-mediated killing)

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Communicated by Zanvil A. Cohn, January 9, 1989

ABSTRACT **Cytotoxic T lymphocytes (CTLs) kill their targets by a contact-dependent mechanism. We investigated the possibility that the CTL membranes themselves could exert direct cytotoxic activity. Murine CTLs that had been fixed with paraformaldehyde retained a slow cytotoxic activity toward various target cells that are also sensitive to another cytokine, tumor necrosis factor (TNF)/cachectin. This cytotoxic activity was neutralized by antibodies specific for TNF. Membrane fractions obtained from CTLs were cytotoxic to TNF-sensitive targets but not to several TNF-resistant cell lines. Immunoblot analysis revealed a membrane protein band of 50–60 kDa from CTLs that reacts with anti-TNF antibodies. The surface localization of this cytokine was further ascertained by flow cytometry, indirect immunofluorescence, and immunoelectron microscopy studies using TNF-specific antibodies. Radioiodination of CTL surface proteins followed by immunoprecipitation with anti-TNF antibodies confirmed the presence of a TNF-related cytokine in the plasma membranes of CTLs that migrated with an apparent molecular mass of 50–60 kDa under disulfide-reducing conditions. This cytokine can be removed from membranes by treatment with detergents but not with high-salt buffers, suggesting that it may be an integral membrane protein.**

Cytotoxic T lymphocytes (CTLs) kill their targets by a contact-dependent mechanism. The nature of this lytic mechanism has remained poorly understood. According to one proposed model, CTL-mediated lysis may occur through a calcium-dependent secretory pathway, involving the assembly of complement-like pores (1–5). Pore formation is mediated by a pore-forming protein (PFP, also termed perforin or cytolysin) found in the cytoplasmic granules of cultured CTLs. This pore-formation pathway is known to be absolutely dependent on calcium. The strict requirement for this type of killing has recently been contested by several observations that have suggested a calcium-independent pathway of killing (6–10). Perforin is absent from several CTL lines that nevertheless display vigorous cytolytic activity (9) and, moreover, killing proceeds in some instances in the absence of extracellular calcium (7–9). Perforin also appears to be absent from primary CTLs generated from mixed lymphocyte reactions (11).

Among other candidate mediators of cytotoxicity, cytotoxins that are immunologically and functionally related to two other well-known cytokines, tumor necrosis factor (TNF or cachectin) (12, 13) and lymphotoxin (14), have been identified in murine (15, 16) and human (17, 18) CTLs. Similar factors have also been described in human natural killer (NK) cells (19, 20). In our laboratory, a soluble TNF/cachectin-like factor was isolated by affinity chromatography using anti-TNF antibodies and was shown to be lytic to a variety of cellular targets (16).

Here, we describe the identification and partial biochemical characterization of a plasma membrane-bound cytotoxin of murine CTLs. This membrane cytotoxin is immunologically related to TNF/cachectin and it mediates lysis only of tumor targets that are also sensitive to TNF. This surface cytotoxin may play a direct role in mediating cytotoxicity during contact between CTLs and their target cells.

MATERIALS AND METHODS

Cell Cultures. The mouse CTL lines CTLL-R8, -1, -2, -A11, and -L3 were maintained in interleukin 2-containing medium, as described (18). Supernatant from rat cells stimulated for 24–36 hr with concanavalin A at 5 μg/ml and phorbol 12-tetradecanoate 13-acetate at 10 ng/ml was used as a source of interleukin 2. Murine fibroblast cell line L929 and a TNF-resistant variant of L929 cells (21) were kindly provided by B. A. Williamson and L. J. Old (Sloan–Kettering Institute, New York). Murine YAC-1 lymphoma and EL-4 thymoma cells were maintained in alpha modified minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum.

Antibodies. Antibodies to murine recombinant TNF (rTNF) were prepared against rTNF obtained as a gift from M. A. Palladino (Genentech). Murine rTNF (50 μg) was reduced and alkylated with dithiothreitol and iodoacetamide, respectively, and boiled in 1% SDS. Booster injections were given every 3 weeks and blood was collected 4 days after each injection.

Subcellular Fractionation and Membrane Preparation. Cells were disrupted by nitrogen cavitation at 2×10^7 cells per ml, as described (22). The nuclei and the organelle-enriched fraction were removed by centrifugation at $800 \times g$ for 15 min and $13,000 \times g$ for 20 min, respectively. Membranes were pelleted at $100,000 \times g$ for 1 hr. The membrane-enriched pellet was washed sequentially with 1 M sodium phosphate buffer (pH 7.4) followed by 0.1 M NaOH. The pellet after high-speed centrifugation ($100,000 \times g$, 1 hr) was extracted twice with diethyl ether and resuspended in Dulbecco's phosphate-buffered saline (PBS). This material was used for cytotoxicity and immunoblot analyses. Protein concentration was measured with a protein assay kit (Pierce).

Immunoblot Analysis, Surface Radiolabeling, and Immunoprecipitation. Cells were lysed in PBS containing 1% Nonidet P-40 (NP-40; Calbiochem–Behring), 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (lysis buffer) and centrifuged at $10,000 \times g$ in a microcentrifuge (Fisher) for 1 min to

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Abbreviations: CTL, cytotoxic T lymphocyte; E/T, effector-to-target; FACS, fluorescence-activated cell sorter; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NP-40, Nonidet P-40; TNF, tumor necrosis factor; rTNF, recombinant TNF.

sediment cell debris. The cell lysate proteins contained in this supernatant or membrane proteins prepared by subcellular fractionation as described above were then reduced with 50 mM dithiothreitol and boiled for 5 min, prior to loading onto a SDS/10% polyacrylamide gel. Gel electrophoresis and immunoblot analysis were done essentially as described (16). The blots were developed with ^{125}I-labeled goat anti-rabbit IgG F(ab')$_2$.

For immunoprecipitation studies, CTLL-R8 cells were first surface-labeled with ^{125}I by the lactoperoxidase method at 1 mCi (37 MBq) of ^{125}I per 10^7 cells (23), which typically yielded 2.2×10^7 cpm per 10^7 cells. Labeled cells were washed with PBS and resuspended in the same buffer to 10^7 per ml and reacted with anti-TNF antiserum or preimmune serum [1:50 (vol/vol) dilution of serum] for 1 hr on ice. After three washes in PBS, cells were resuspended in lysis buffer. After sitting on ice for 30 min, cell debris and unbroken cells were sedimented by centrifugation in a microcentrifuge ($10,000 \times g$; 1 min), and the supernatant was incubated with protein A-agarose (Polysciences) for an additional 1 hr followed by sedimentation in the microcentrifuge. After three cycles of alternate washes with PBS/0.55 M NaCl/0.5% NP-40 and PBS, the immunoprecipitate was resuspended in gel sample buffer (2% SDS/25 mM dithiothreitol/20% glycerol) and boiled for 5 min prior to gel electrophoresis and autoradiography. Autoradiography was performed with an intensifying screen at −70°C.

Immunoelectron Microscopy. CTLL-R8 cells were washed in PBS, resuspended to 10^6 per ml in PBS/1% bovine serum albumin (BSA) and incubated on ice for 30 min. The cells were sedimented in a microcentrifuge, washed three times in PBS, and then incubated with either anti-rTNF antiserum or preimmune serum (1:50 dilution in PBS/1% BSA) for 1 hr on ice. After the cells were washed in PBS and incubated with protein A-coupled colloidal gold (1:25 dilution in PBS/0.1% BSA; Janssen Pharmaceutica) for 1 hr on ice, they were washed and processed for electron microscopy as described (24). The samples were examined and photographed on a JEOL 100 CX electron microscope (EM). The gold particles bound to the plasma membranes of individual cell profiles were counted on the EM. No more than 20 cells per EM grid were used for counting, and profiles were selected for counting that demonstrated both a nucleus and an intact plasma membrane.

Cell Viability Assays. CTLL-R8 and CTLL-1 cells were fixed with 1% paraformaldehyde in PBS for 30 min, washed in PBS, and then incubated at 37°C for 12 hr, after which they were again washed with α-MEM. L929 cells (2×10^4) were added to each microtiter well in triplicates and different effector-to-target (E/T) ratios were achieved by varying the number of CTLL cells. The final volume of the mixture was 200 μl per well. After incubation for specified periods of time, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] dye reduction assay (25) was performed as described (16). For neutralization experiments, serially diluted rTNF-specific antiserum or preimmune serum was included during the MTT assay.

Fluorescence-Activated Cell Sorter (FACS) Analysis. Cells were stained with either rTNF-specific antiserum or preimmune serum at 1:100 dilution, followed by fluorescein-conjugated goat anti-rabbit IgG F(ab')$_2$ (Tago). For Fig. 2, 5000 cells (with the exception for preimmune serum-treated CTLL-R8 of 3000 cells) were analyzed on a FACScan (Becton Dickinson).

RESULTS

Cytotoxic Activity Is Associated with Membranes of CTLs. We reasoned that a cytotoxic activity present on the surface of CTLs could be tested by studying metabolically inert cells. CTLL-R8 cells were fixed with paraformaldehyde prior to their incubation with target cells. Paraformaldehyde-fixed effector cells remained cytotoxic in a dose-dependent manner to murine fibroblast L929 cells, as measured by the MTT dye reduction assay (Fig. 1*a*). The fixed effector cells interfered little with this assay, accounting for <10% of the MTT dye taken up by the cells even at the highest CTL numbers used, indicating that the fixed cells must have been metabolically inactive. The cytotoxicity mediated by fixed cells showed a slow time course, taking several hours for detection of cell damage (Fig. 1*a*). The cytotoxic reaction could not be attributed to lytic factor(s) that had leaked from fixed cells since, in all experiments, fixed cells were allowed to sit in medium for at least 12 hr and were then washed in fresh

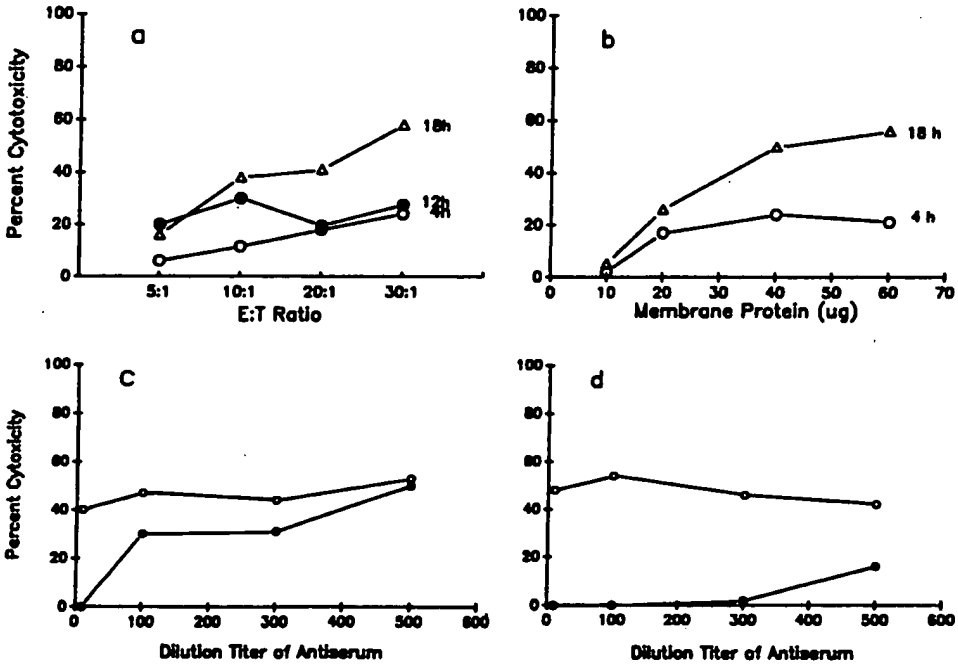


FIG. 1. (*a*) Cytotoxic activity of paraformaldehyde-fixed CTLL-R8 cells using L929 fibroblasts as targets. Cell mixtures were incubated at the given E/T ratios for 4, 12, or 18 hr, after which cytotoxicity was determined by the MTT assay. (*b*) Dose–response curve for cytotoxicity to L929 cells mediated by membrane proteins isolated from CTLL-R8 cells. (*c* and *d*) Neutralization of cytotoxicity to L929 cells associated with paraformaldehyde-fixed CTLL-R8 cells (*c*) and with CTLL-R8 membrane proteins (*d*) by rTNF-specific antiserum (●) and preimmune control serum (○). In *c* and *d*, an 18-hr end point was used for the MTT assay. An E/T ratio of 10:1 was used in *c*, and 100 μg of membrane protein per ml was used in *d*. The background levels of cytotoxicity were subtracted from each data point.

medium before their use in cytotoxicity assays. In parallel experiments, paraformaldehyde-fixed L929, YAC-1 lymphoma, and P815 mastocytoma cells did not induce any measurable level of cytotoxicity even at E/T ratios of 30:1 (Table 1).

Membrane preparations from CTLL-R8 cells were next enriched by a differential centrifugation procedure. Membrane sheets and vesicles that had been depleted of nuclei and cell organelles (electron micrographs not shown) were next washed sequentially with high salt (1 M phosphate) and high pH (0.1 M NaOH) solutions to remove attached peripheral membrane proteins. The final membrane pellet obtained after high-speed centrifugation was found to be cytotoxic to L929 cells in a concentration-dependent manner (Fig. 1*b*). The supernatants collected from all the membrane washes were devoid of any cytotoxic activity, ruling out the possibility that the cytotoxic factor(s) could have been attributed to peripheral membrane proteins.

Membrane Cytotoxin Is Immunologically Related to TNF/Cachectin. The close resemblance between the membrane lytic factor and the macrophage cytokine TNF/cachectin was established as follows. Killing of L929 target cells by fixed CTLL-R8 was effectively blocked by a polyclonal antiserum derived against rTNF (Fig. 1*c*). This antiserum also blocked the cytotoxicity mediated by membrane-enriched fractions from R8 cells (Fig. 1*d*). Control experiments done with preimmune serum failed to yield any inhibitory response. A TNF-resistant variant of L929 cells (21) was not killed by fixed R8 cells even at an E/T ratio of 30:1 and with MTT assays performed up to 30 hr of incubation (Table 1). Fixed R8 cells also killed TNF-sensitive WEHI-164 fibrosarcoma cells but not the TNF-insensitive YAC-1, P815 mastocytoma, or EL-4 cells (Table 1). Using rTNF-specific antibodies, the immunologic crossreactivity between the membrane factor and TNF was also demonstrated by FACS analysis of two CTL lines (Fig. 2) and by indirect immunofluorescence staining (which revealed a diffuse stain on the surface of cells that was absent from cells stained with preimmune serum; data not shown). A heterogeneity of cell distribution of surface staining was observed within the same cloned population of CTLs as determined by FACS, reflecting perhaps differences of surface expression of the antigen that might be associated with the cell cycle.

To further ascertain that a portion of this cytotoxin was indeed associated with the cell surface, CTLL-R8 cells were surface-labeled with anti-rTNF antibody followed by protein A-conjugated colloidal gold. Electron micrographs showed gold particles bound to cells treated with specific antiserum (Fig. 3 *a* and *b*) but not to preimmune serum-treated cells (Fig. 3*c*). Quantitation of the number of gold particles bound per individual cell profile revealed that the number of particles associated with antiserum-treated cells ranged from 4 to 52 with a distribution as shown in Fig. 3*d*. In contrast, preimmune serum-treated control cells were virtually devoid of particles (see legend to Fig. 3).

Immunoblot analysis was next performed on detergent-solubilized proteins derived from membrane-enriched fractions and total CTLL-R8 lysate proteins. A single protein band migrating with apparent molecular mass of 50–60 kDa was found in both preparations to react with TNF-specific antibodies (Fig. 4*a*, lanes 1 and 2). Preimmune serum controls failed to reveal any reactive band (lanes 3 and 4). The relative distribution of the antibody label, measured by the intensities of the radioactivity associated with the blots, showed that the membrane-associated form accounted for only 25% of the total cell reactivity (Fig. 4*b*). Since plasma membranes comprise only a portion of the total pool of cellular membranes, this figure may represent an upper estimate of the total amount of cytotoxin associated with the cell surface.

The surface location of the membrane factor was also demonstrated by immunoprecipitation studies. CTLL-R8 cells were surface-iodinated with ^{125}I by the lactoperoxidase technique. A single protein band with molecular mass ranging from 50 to 60 kDa was immunoprecipitated from surface-labeled cells with TNF-specific antibodies but not with preimmune control serum (Fig. 5).

In parallel experiments, noncytotoxic YAC-1 and EL-4 cells were found to be devoid of any membrane-associated

Table 1. Cytotoxic activity of paraformaldehyde-fixed CTLL-R8 and non-CTL cells

Effector cells	Target cells	% cytotoxicity 5:1	% cytotoxicity 30:1
CTLL-R8	L929 (TNF-sensitive)*	16	58
CTLL-R8	L929 (TNF-resistant)†	3	6
CTLL-R8	WEHI-164	33	84
CTLL-R8	EL-4	0	4
CTLL-R8	P815	2	3
CTLL-R8	YAC-1	6	9
P815	L929 (TNF-sensitive)*	2	5
YAC-1	L929 (TNF-sensitive)*	0	4

CTLL-R8, P815, and YAC-1 cells were fixed with paraformaldehyde and mixed with the indicated target cells at E/T ratios of 5:1 and 30:1. Cytotoxicity was determined by the MTT assay after 18 hr of incubation. Similar results were obtained in 30-hr assays.
*A TNF-sensitive L929 fibroblast subclone.
†A TNF-resistant subclone.

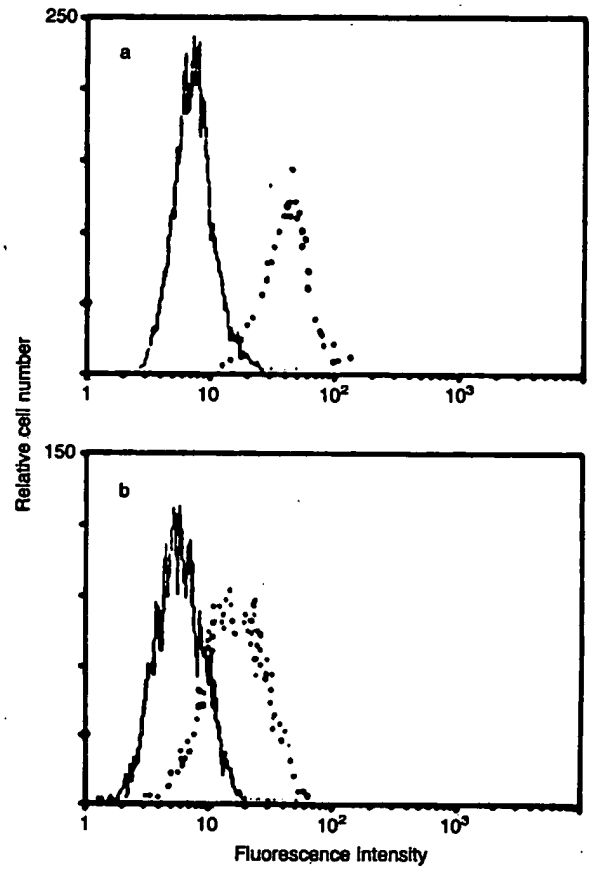


FIG. 2. FACS analysis of CTLL-R8 (*a*) and CTLL-1 (*b*) cells with anti-rTNF antibodies. Cells were stained with either rTNF-specific antiserum (---) or preimmune serum (—) at 1:100 dilution, followed by fluorescein-conjugated goat anti-rabbit IgG F(ab')$_2$.

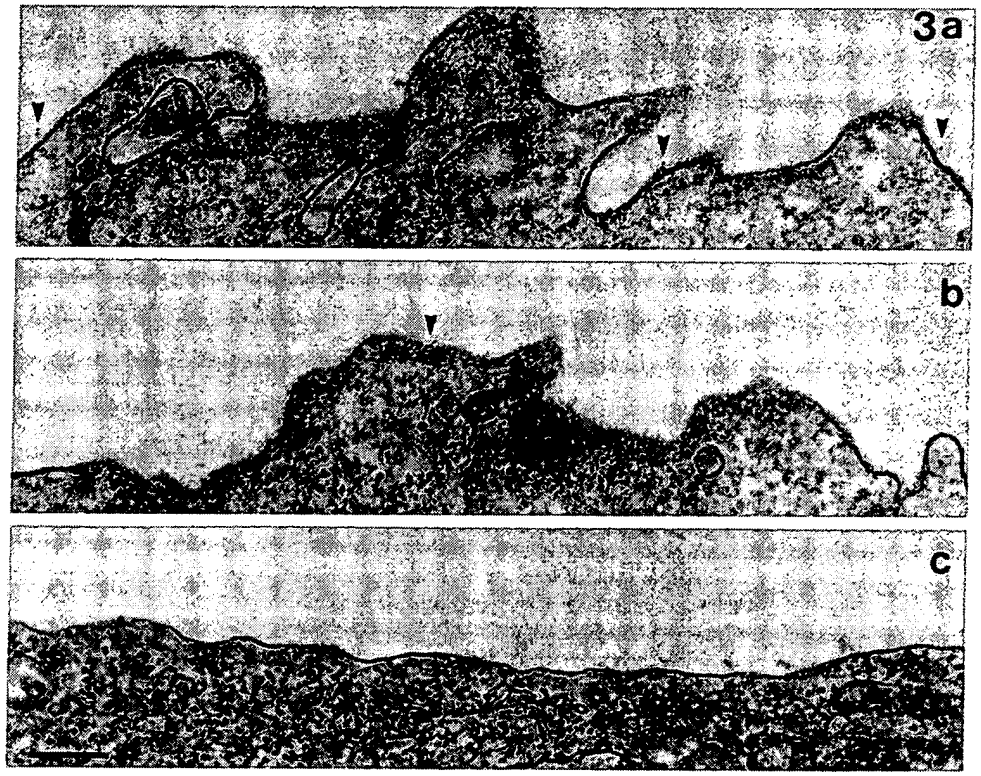
cytotoxicity (Table 1) or TNF/cachectin-like cytotoxin (data not shown). On the other hand, the membrane cytotoxin was also identified functionally and immunologically in four other murine CTL lines (CTLL-1, -2, -A11, and -L3).

DISCUSSION

Because CTLs require a close contact with their target cells to mediate lysis, we examined whether CTL membranes are cytotoxic to target cells. We found that paraformaldehyde-fixed cells as well as membrane fractions extracted from murine CTLs exert a slow cytotoxic activity on various target cells and that this lytic activity can be blocked completely by anti-TNF antibodies. Immunochemical analysis revealed a TNF-related, membrane cytotoxin that migrates as a 50- to 60-kDa protein under disulfide-reducing conditions. Since this protein could be extracted from membranes by detergent but not by high salt and high pH washes, it is possible that this membrane-bound cytotoxin is an integral membrane protein. The membrane-bound form described here may be related structurally to a soluble cytosolic cytotoxin of the same molecular mass that has previously been described in murine CTLs (16). Unlike the cytosolic form, however, the membrane-bound cytotoxin lyses only TNF-susceptible targets.

Several recent studies have also implied a membrane localization for the macrophage TNF/cachectin (26–29). The present study describes a membrane-bound cytotoxin of CTLs that is related to the macrophage TNF. It remains to be seen whether this membrane-bound cytotoxin plays any active role in cell killing; perhaps, like TNF/cachectin, it may also function as a pleiotropic lymphokine. In this respect, it should be noted that although membranes of lymphocyte populations have previously been implicated in cytotoxic reactions (30), a mechanistic role for membranes in T-cell-mediated killing was not supported by a subsequent study (31). Another issue of concern is the time course of killing mediated by CTL membranes, which is significantly slower than that associated with killing by viable CTLs. In fact, the activity described here resembles the so-called natural cytotoxic (NC) activity described for effector spleen cells (32). Thus, still other unidentified factors may be involved in lymphocyte-mediated killing.

In addition to the pore-formation model proposed to explain lymphocyte-mediated killing, an internal disintegration or induced suicide model has been suggested whereby lymphocytes are thought to activate a program of self-death within the target (33, 34). Accordingly, the DNA of the target cell has been shown to undergo a rapid onset of fragmentation



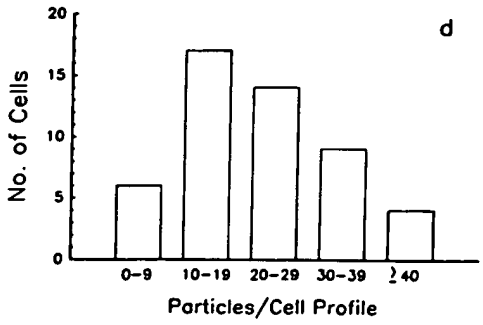


FIG. 3. (*a–c*) Electron micrographs of CTLL-R8 cells surface-labeled with immunogold. CTLL-R8 cells were stained with either rTNF-specific antiserum (*a* and *b*) or preimmune serum (*c*). Arrowheads indicate gold particles. (×55,000. Bar = 0.2 μm.) (*d*) Histogram of gold particles bound per cell profile on CTLL-R8 cells labeled with anti-TNF. The antiserum-treated cells had an average of 22.9 ± 11.3 particles per cell profile (mean ± SEM of n = 50 cells), whereas preimmune serum-treated control cells had 0.13 ± 0.34 particles per cell profile (n = 30 cells).

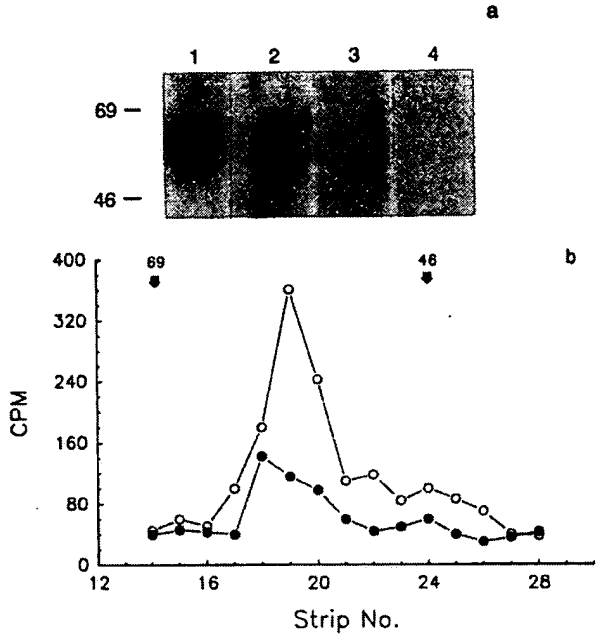


FIG. 4. Immunoblot analysis of CTLL-R8 cell and membrane proteins with anti-rTNF antibodies. (*a*) Total cell lysates (lanes 1 and 3) and membrane proteins (lanes 2 and 4) from 10^5 cell equivalents of CTLL-R8 were electrophoresed in each lane and immunoblotted with either anti-rTNF antiserum (lanes 1 and 2) or preimmune control serum (lanes 3 and 4). The positions of albumin (69 kDa) and ovalbumin (46 kDa) are indicated at left. (*b*) Distribution of anti-TNF antibody label in total cell lysate and in the membrane fraction. The blot shown in *a* was cut into 1-mm strips. Radioactivity associated with each strip was plotted as a function of distance from the top of the blot. Integration of the peaks revealed that the membrane fractions accounted for 25% of the total cell radioactivity.

into repeat units of about 200 base pairs (33, 35, 36). The mediators responsible for this pathway have not been identified, and preliminary studies have shown that purified perforin does not induce DNA fragmentation (unpublished data). It will be important in the near future to assess rigorously the role of putative mediators, including the membrane cytotoxin described here, in the different pathways of cell killing.

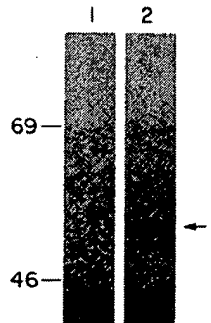


FIG. 5. Immunoprecipitation of surface-labeled proteins with anti-TNF antibodies. CTLL-R8 cells were surface-labeled with ^{125}I, incubated with either preimmune serum (lane 1) or polyclonal anti-TNF antiserum (lane 2), lysed with NP-40, and precipitated with protein A-agarose. Approximately 5×10^6 cell equivalents were applied per lane to a SDS/10% polyacrylamide gel. The specific protein band is shown by an arrow. Autoradiographic exposure time was 5 days.

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Targeting of cytokine gene expression to malignant melanoma cells using tissue specific promoter sequences.

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BACKGROUND: Transduction of tumor cells in vitro with cDNAs encoding various cytokines and/or immune accessory molecules has been shown to diminish or eliminate tumorigenicity when such cells are returned in vivo to syngeneic animals. One method being explored for in situ gene therapy is to use tissue-specific promoters to direct expression of the therapeutic genes to the tumour cells. DESIGN: This study used the 5' flanking region of the murine tyrosinase gene to direct expression of three different cytokine genes [murine interleukin 2 (IL-2), IL-4 and macrophage colony-stimulating factor (M-CSF)] specifically to murine melanoma cells. RESULTS: Expression of the IL-2 gene, from 2.5 kbp of the 5' flanking region of the murine tyrosinase gene, was detected in 11 out of 55 puromycin-resistant B16 clones isolated after transfection. The highest producing clone secreted 2000 pg/ml/10(6) cells/48 hours as determined by enzyme-linked immunosorbent assay. The IL-2 was tested for biological activity by its ability to stimulate proliferation of the IL-2 dependent CTLL cell line. No detectable level of IL-2 expression occurred in 58 clones of drug-resistant NIH 3T3 cells derived after transfection with the same construct. Similar results were obtained following transfection of these two cell lines with the tyrosinase-IL-4 minigene construct. Expression of IL-2 in the murine melanoma cells completely abrogated their tumorigenicity in syngeneic mice. However, progressively growing tumours were produced from clones in which the IL-2 gene was no longer expressed (as determined by reverse transcriptase polymerase chain reaction). Direct injection of DNA encoding cytokine genes, expressed from the tyrosinase promoter, into established B16 melanomas in syngeneic mice resulted in gene expression within the tumour mass. While no change in tumour growth was observed following such treatment, the results demonstrate that direct injection of naked DNA into a neoplasm can result in uptake and expression of cytokine

genes up to 16 days post-injection. CONCLUSION: The use of tissue-specific promoters can limit expression to the required target cell, while the choice of appropriate gene should result in an alteration in tumour burden.

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